



**University of Dundee**

**MTHFR promoter methylation might mitigate the effect of smoking at the level of LINE-1 in cleft lip tissues**

Khan, Mohammad Faisal J.; Little, Julian; Mossey, Peter A.; Butali, Azeez; Autelitano, Luca; Meazzini, Maria C.

*Published in:*  
Birth Defects Research

*DOI:*  
[10.1002/bdr2.1959](https://doi.org/10.1002/bdr2.1959)

*Publication date:*  
2021

*Document Version*  
Peer reviewed version

[Link to publication in Discovery Research Portal](#)

*Citation for published version (APA):*

Khan, M. F. J., Little, J., Mossey, P. A., Butali, A., Autelitano, L., Meazzini, M. C., & Rubini, M. (2021). MTHFR promoter methylation might mitigate the effect of smoking at the level of LINE-1 in cleft lip tissues: A preliminary study. *Birth Defects Research*, 113(20), 1463-1469. <https://doi.org/10.1002/bdr2.1959>

**General rights**

Copyright and moral rights for the publications made accessible in Discovery Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from Discovery Research Portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain.
- You may freely distribute the URL identifying the publication in the public portal.

**Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.



**MTHFR promoter methylation might mitigate the effect of smoking at the level of LINE-1 in cleft lip tissues – a preliminary study**

Journal:	<i>Birth Defects Research</i>
Manuscript ID	BDR-21-0068.R1
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	18-Jul-2021
Complete List of Authors:	Khan, Mohammad Faisal Jamal; University of Ferrara, Department of Neuroscience and Rehabilitation, Section of Medical Biochemistry, Molecular Biology and Genetics Little, Julian; University of Ottawa, School of Epidemiology and Public Health Mossey, Peter; University of Dundee, Craniofacial Development at the World Health Organization–collaborating Centre for Oral and Craniofacial Research, Dental Hospital and School Butali, Azeez; University of Iowa, Department of Oral Pathology, Radiology and Medicine, College of Dentistry Autelitano, Luca; University of Milan, Smile House Milan, Regional Centre for Orofacial Clefts and Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo Hospital Meazzini, Maria Cristina; University of Milan, Smile House Milan, Regional Centre for Orofacial Clefts and Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo Hospital Rubini, Michele; University of Ferrara, Department of Neuroscience and Rehabilitation, Section of Medical Biochemistry, Molecular Biology and Genetics
Key Words:	Non-syndromic cleft lip with or without cleft palate, DNA methylation, MTHFR c.677C>T, MTHFR promoter methylation, LINE-1

SCHOLARONE™  
Manuscripts

This is the peer reviewed version of the following article: Khan, M.F.J, et al. (2021) 'MTHFR promoter methylation might mitigate the effect of smoking at the level of LINE-1 in cleft lip tissues: A preliminary study', Birth Defects Research 13(20), pp. 1463-1469, which has been published in final form at <https://doi.org/10.1002/bdr2.1959>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

1  
2  
3  
4 **1** *MTHFR* promoter methylation might mitigate the effect of smoking at the level of  
5  
6 **2** *LINE-1* in cleft lip tissues – a preliminary study  
7

8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

3 Mohammad Faisal J Khan, Julian Little, Peter A Mossey, Azeez Butali, Luca Autelitano,  
4 Maria C Meazzini, Michele Rubini

5 **Mohammad Faisal J. Khan:** Department of Neuroscience and Rehabilitation, Section  
6 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara,  
7 Ferrara, Italy. Email: khnmmm@unife.it

8 **Julian Little:** School of Epidemiology and Public Health, University of Ottawa, Ottawa,  
9 Ontario, Canada. Email: jlittle@uottawa.ca

10 **Peter A. Mossey:** Craniofacial Development at the World Health Organization–  
11 collaborating Centre for Oral and Craniofacial Research, Dental Hospital and School,  
12 University of Dundee, Dundee, Scotland. Email: p.a.mossey@dundee.ac.uk

13 **Azeez Butali:** Department of Oral Pathology, Radiology and Medicine, College of  
14 Dentistry, University of Iowa, Iowa City, IA, USA. Email: azeez-butali@uiowa.edu

15 **Luca Autelitano:** Smile House Milan, Regional Centre for Orofacial Clefts and  
16 Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo  
17 Hospital, University of Milan, Milan, Italy. Email: luca.aute@gmail.com

**Maria C Meazzini:** Smile House Milan, Regional Centre for Orofacial Clefts and  
Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo  
Hospital, University of Milan, Milan, Italy. Email: cmeazzini@yahoo.it

18 **Michele Rubini:** Department of Neuroscience and Rehabilitation, Section of Medical  
19 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.  
20 Email: rub@unife.it

21  
22 **Corresponding author.**

23 **Mohammad Faisal J. Khan,** Department of Neuroscience and Rehabilitation, Section  
24 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Via  
25 Fossato di Mortara 74 I-44121, Ferrara, Italy. Email: khnmmm@unife.it

26  
27 **Funding information.** This study was partly supported by FAR-2018 grant from University  
28 of Ferrara and from Department of Biomedical and Specialty Surgical Sciences, Section  
29 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Italy,  
30 Grant/Award Number: 62040 and 0039804

1  
2  
3  
4 **Abstract**  
5

6 **Background:** The medial and maxillary aspects of the upper lip originate at separate  
7 embryonic stages and therefore may experience different maternal exposure patterns  
8 which may affect methylation. Based on this hypothesis, we investigated the level of  
9 methylation of the methylene tetrahydrofolate reductase promoter gene (*mMTHFR*) in  
10 tissues from cleft lip, and *mMTHFR* levels by *MTHFR* c.677C>T genotype. We further  
11 investigated whether *mMTHFR* mitigates the effect of smoking on long interspersed  
12 nuclear element (LINE-1) methylation in these tissues.  
13  
14  
15  
16  
17  
18

19 **Methods.** DNA extracted from medial and lateral tissues of 26 infants with non-  
20 syndromic cleft lip with or without cleft palate (nsCL/P) was bisulfite converted and  
21 *mMTHFR* was measured on a pyrosequencer. LINE-1 methylation and *MTHFR*  
22 c.677C>T genotype data were obtained in our previous study.  
23  
24  
25

26 **Results.** There was no substantial difference in *mMTHFR* ( $p=0.733$ ) and LINE-1  
27 ( $p=0.148$ ) between the two tissues. *mMTHFR* was not influenced by *MTHFR* c.677C>T  
28 genotype, but there was suggestive evidence that the difference was larger among infants  
29 exposed to maternal smoking compared to non-exposed. LINE-1 methylation differences  
30 were significant ( $p=0.025$ ) in infants born to non-smoking mothers, but this was not  
31 apparent ( $p=0.872$ ) in infants born to mothers who smoked. Our Pearson's correlation  
32 analysis suggested a weak inverse association between *mMTHFR* and LINE-1 ( $r=-0.179$ ;  
33  $p=0.381$ ).  
34  
35  
36  
37  
38  
39  
40

41 **Conclusion.** Our preliminary observation of differences in patterns of *mMTHFR* levels  
42 in lip tissue suggests the interplay of gene and environment in establishment of  
43 methylation in tissues at both sides of cleft lip. This requires investigation in a larger  
44 cohort, integrated with metabolic assessment.  
45  
46  
47

48 **Keywords:** Non-syndromic cleft lip with or without cleft palate, DNA methylation,  
49 *MTHFR* c.677C>T, LINE-1, *MTHFR* promoter methylation  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## 59 Introduction

60 Orofacial clefts (OFCs) are collectively among the most common human congenital  
61 anomalies that can occur in isolation or as part of a syndrome (Mossey et al., 2011). Some  
62 environmental and multiple genetic risk factors have been identified for non-syndromic  
63 form of OFCs (Leisle & Marazita, 2013; Khan et al., 2018a, Mossey et al., 2017; Raut et  
64 al., 2019; Johnson & Little, 2008; Little, Cardy, & Munger, 2004) but the causes of these  
65 defects remain largely unknown.

66 OFCs develop in early life, when the embryo is extremely susceptible to perturbation of  
67 the in-utero environment (Dixon, Marazita, & Beaty, 2010). Among environmental  
68 factors, tobacco smoking has been found to influence facial morphology (Xuan et al.,  
69 2016), and is reported to be the most consistent and strongest risk factor for OFCs (Raut  
70 et al., 2019). Such perturbation of the early life environment affects developmental  
71 programming in the embryo, with sustained changes potentially detectable in tissues from  
72 medial nasal and maxillary sides of the upper lip in cases with non-syndromic cleft lip  
73 with or without cleft palate (nsCL/P) as observed by our group in recent studies (Khan et  
74 al., 2018b, Khan et al., 2018c, Khan et al., 2019a, Khan et al., 2019b).

75 Methylene tetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the one-  
76 carbon cycle, a pathway that is critical to metabolism of folate. Folate is a specific nutrient  
77 involved in development of craniofacial structures (Jiang, Bush, & Lidral, 2006) and  
78 provides methyl group for DNA methylation (Sinclair et al., 2007). MTHFR activity is  
79 mainly regulated by the combination of two mechanistic aspects – 1) variants within the  
80 gene that essentially acts at the level of enzyme activity and specificity; 2) methylation  
81 of the gene promoter that mainly affects level of expression. Both the c.677C>T  
82 (rs1801133) and c.1298A>C (rs1801131) variants of the *MTHFR* gene have been  
83 demonstrated to reduce enzyme activity (Liew & Gupta, 2015; van der Put et al., 1998).  
84 The effect of both these variants have been investigated in relation to nsCL/P in the index  
85 child, and/or one or both parents but the results from these studies have been inconclusive  
86 (Rai, 2018; Mossey, & Little, 2002; Reutter et al., 2008; Shaw, Todoroff, Finnell, Rozen,  
87 & Lammer, 1999; Zhou et al., 2020). Our group; however, considered an alternative  
88 mechanism involving DNA methylation to decipher the role of *MTHFR* gene variants in

89 nsCL/P, and found that a variant in *MTHFR* gene plays a role in the establishment of  
90 methylation in cleft lip tissues (Khan et al., 2019b).

91 We know that methylation within *MTHFR* promoter (*mMTHFR*) contributes to variation  
92 in *MTHFR* protein activity similar to that conferred by *MTHFR* variants (Coppede,  
93 Denaro, Tannorella, & Migliore, 2016), and has been shown to contribute to many  
94 developmental (Asim, Agarwal, Panigrahi, Sai, yed, & Bakshi, 2017; Coppede et al.,  
95 2016) and pregnancy related disorders (Ge et al., 2015; Mishra et al., 2019). However, to  
96 our knowledge, there appears to be no evidence of information regarding the methylation  
97 profile of *mMTHFR*, or the contribution of *MTHFR* variants to *MTHFR* methylation level  
98 in nsCL/P. Therefore, we undertook this preliminary study to assess the level of  
99 *mMTHFR*, and further investigate the relationship between *MTHFR* c.677C>T variant  
100 and *mMTHFR* utilizing tissues from medial and lateral aspects of the upper lip in  
101 individuals with nsCL/P.

102 *mMTHFR* could also be involved in differences in regulation of methylation repair  
103 activity and hence might contribute to individual differences by altering enzyme activity.  
104 This could either affect the availability of activated methyl group or increase the rate of  
105 loss of methylation (over time) in response to exposures associated with demethylation  
106 such as cigarette smoking (Beach et al., 2017). Alternatively, when *MTHFR* is more  
107 active, the availability of methyl group is more likely enhanced, potentially alleviating  
108 the impact of exposures such as smoking that would otherwise cause demethylation  
109 (Beach et al., 2017; Stover, 2009). Intrigued by this concept, we examined whether  
110 *mMTHFR* in the indexed infant could mitigate the adverse effects of active maternal  
111 smoking exposures, and potentially be reflected as changes in LINE-1 methylation level  
112 – widely accepted to be a proxy for overall genomic DNA methylation content (Lisanti  
113 et al., 2013).

## 114 **Materials and Methods**

115 Tissue samples from the medial and lateral sides of cleft lip were collected from 26 cases  
116 with nsCL/P that were recruited between 2016 and 2018 in the Centre for Orofacial Clefts  
117 and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy (PENTACLEFT: prot. no.  
118 08–2011). Our sample included 13 female and 13 male cases. Fifteen cases had cleft lip

1  
2  
3  
4 119 and 10 had cleft lip and palate (phenotypic data missing for one case – due to  
5 120 mismatching). Among the mothers of these 26 cases, fifteen were non-smokers and eight  
6 121 actively smoked during the periconceptional period – 3 months before to 3 months after  
7 122 conception (smoking data missing for three mothers – due to non-response on survey).

11  
12 123 The DNA extracted from tissues were bisulfite converted and methylation of the *MTHFR*  
13 124 gene promoter was measured using PyroMark Q96 predesigned CpG assay  
14 125 (#PM00000091) on a PyroMark Q96 ID pyrosequencer (Qiagen GmbH, Hilden,  
15 126 Germany), with minor modification of the method previously described (Khan et al.,  
16 127 2018b). Briefly, the amplification of bisulfite converted DNA was performed by PCR  
17 128 with *MTHFR* primer, and pyrosequencing done using *MTHFR* specific sequencing  
18 129 primers. The predesigned assay contained the following sequence 5'-  
19 130 GGTCAGTACCGATGGGGGCGAGGAYACGGGC-3' (prior to bisulfite  
20 131 conversion) including 3 CpG sites to assess in promoter region of *MTHFR*. The  
21 132 nucleotide dispensation order was:  
22 133 TGTCATGATGATATCGAGTGGTCGAGATATCG. LINE-1 methylation and  
23 134 *MTHFR* c.677C>T genotype data for this cohort were obtained in our previous study  
24 135 (Khan et al., 2019b). The Kolmogorov-Smirnov test showed that the data were normally  
25 136 distributed, hence, parametric comparison of within and between samples were performed  
26 137 using Student's *t*-test. In addition, we report parametric effect size estimate (Hedge's  $g_s$ )  
27 138 associated with independent sample Student's *t*-test, and Pearson's correlation to  
28 139 determine relationship between m*MTHFR* and LINE-1 (Pautz, Olivier, & Steyn, 2018;  
29 140 McLeod, 2019). Considering the total of 26 cases included in this study, we calculated  
30 141 statistical power using G\*POWER software 3.1.9.2 version (Faul, Erdfelder, Lang, &  
31 142 Buchner, 2007). Considering a two-tailed *t*-test and an equally serious  $\alpha$  &  $\beta$  error ( $\beta/\alpha =$   
32 143 1), an effect size  $d_z$  of 0.2, we calculated the power (1- $\beta$ -error) of 0.60.

## 33 144 **Results**

34 145 We found a non-significant difference of 1.3% in m*MTHFR* between medial and lateral  
35 146 tissues ( $p=0.733$ ; Table 1). m*MTHFR* was not influenced by *MTHFR* c.677C>T genotype  
36 147 (Table 2). Similarly, LINE-1 methylation was not significantly different (1.7%) across  
37 148 tissues in this cohort ( $p=0.148$ ; Table 1).



1  
2  
3  
4 149 To examine the role of *mMTHFR* as a source in compensating for the effect of smoking  
5  
6 150 on LINE-1 methylation level, we compared the level of methylation in *MTHFR* and  
7  
8 151 LINE-1 in medial and lateral tissues between infants born to mothers who smoked in the  
9  
10 152 periconceptual period and infants of non-smoking mothers. Among infants exposed to  
11  
12 153 smoking, the difference in *mMTHFR* was larger (6.1%) but showed a lowered level of  
13  
14 154 methylation ( $p=0.293$ ; Table 1) compared to infants born to non-smoking mothers in  
15  
16 155 whom *mMTHFR* was similar ( $\sim 38\%$ ) in the two tissues ( $p=0.866$ ; Table 1). Interestingly,  
17  
18 156 LINE-1 methylation differences were significant ( $p=0.025$ ) in infants born to non-  
19  
20 157 smoking mothers; this however, was not apparent ( $p=0.872$ ) in infants born to mothers  
21  
22 158 who smoked, with medial and lateral tissues showing equal level (72%) of methylation  
23  
24 159 (Table 1).

25  
26 160 Comparisons between these groups were non-significant for both *mMTHFR* ( $p=0.554$ )  
27  
28 161 and LINE-1 ( $p=0.209$ ). We also calculated the effect size (ES) for between comparisons  
29  
30 162 and found a small ES for *mMTHFR* ( $gs=0.26$ ). While LINE-1 showed medium ( $gs=0.56$ )  
31  
32 163 ES, our Pearson's correlation analysis suggested a weak inverse association between  
33  
34 164 *mMTHFR* and LINE-1 ( $r= -0.179$ ;  $p=0.381$ ).

## 35 36 37 165 **Discussion**

38  
39 166 In this preliminary study, we found a small non-significant difference in *mMTHFR* and  
40  
41 167 LINE-1 methylation across medial and lateral tissues. A difference in *mMTHFR* was  
42  
43 168 observed in infants of mothers who smoked but not among infants born to non-smoking  
44  
45 169 mothers. By contrast, a significant difference in LINE-1 methylation was apparent in  
46  
47 170 infants born to non-smoking mothers, but not in infants born to smoking mothers.

48  
49 171 Our observation of small changes in methylation in *mMTHFR* and LINE-1 is compatible  
50  
51 172 with reports that the magnitude of epigenetic effect associated with exposure in children  
52  
53 173 is generally small; large changes may not be compatible with continued development  
54  
55 174 (Breton et al., 2017). Hence, a small imbalance in methylation in progeny cells of the  
56  
57 175 medial and maxillary tissues might result in an apparently small distinction between  
58  
59 176 sufficient and insufficient methylation. Insufficient methylation might in turn interfere  
60  
177 with the fusion process, so leading to the development of a cleft lip. Persistence of this  
178 small imbalance throughout pregnancy and into the postnatal period would be manifested



1  
2  
3  
4 179 as differences in tissues from medial and lateral side of cleft lip. Such a difference could  
5  
6 180 therefore provide insight into epigenetic effects of early life environmental exposures  
7  
8 181 (Richmond et al., 2017).  
9

10 182 The pattern of *mMTHFR* levels suggests that nearly equal levels of folate are available  
11 183 for tissues developing at distinct embryonic periods, but the level of availability could  
12 184 likely be influenced by external factors such as smoking (Nafee, Farrell, Carroll, Fryer,  
13 185 & Ismail, 2008). Importantly, we found lower *mMTHFR* in the smoking group that  
14 186 suggests an increase in folate availability. This increased folate availability might provide  
15 187 methyl-group to mitigate/overcome the effect of smoking resulting in the observation of  
16 188 nearly equal levels of LINE-1 methylation in the medial and lateral tissues (72%). The  
17 189 lower *mMTHFR* level in the smoking group further suggests smoking-associated  
18 190 demethylation at a single gene promoter, whereas there was little difference in global  
19 191 methylation. This is in line with the suggestion that small changes in global methylation  
20 192 of developing tissues might have substantial effects in the longer term (Breton et al.,  
21 193 2017). Pearson's correlation analysis showed a weak association between *mMTHFR* and  
22 194 LINE-1. This provides some support for a role of *mMTHFR* in moderating epigenetic  
23 195 response to smoking, and our previous findings that lip tissues are highly responsive to  
24 196 maternal environmental exposures (Khan et al., 2018b). We acknowledge that our results  
25 197 are based on small sample size, because collecting tissues from the cleft cases presents  
26 198 considerable challenges (Stock et al., 2016). We did not correct for multiple comparisons  
27 199 because reducing the risk of type I error can be at the expense of increasing type II error,  
28 200 and because of the preliminary nature of the study, identifying hypotheses for further  
29 201 investigation (Perneger 1998; Armstrong, 2014). A limitation of using tissue from the  
30 202 clefts is the difficulty of obtaining an appropriate reference group from which lip tissue  
31 203 samples could be collected. This problem arises from concerns about ethical issues and  
32 204 selection bias and is highly likely to be encountered in other studies.

33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51 205 Our result is consistent with previous reports involving a large number of healthy Italian  
52 206 participants showing no association of *MTHFR* c.677C>T with *mMTHFR* indicating that  
53 207 c.677C>T variant does not act as a *cis* regulatory element to regulate its own gene  
54 208 promoter (Piras et al., 2020; Coppede et al., 2019; Ni et al., 2017), although there are  
55 209 reports that *MTHFR* c.677C>T genotype influences *mMTHFR* (Mandaviya et al. 2017;  
56  
57  
58  
59  
60

1  
2  
3  
4 210 Nash et al., 2019). Accordingly, we found that *mMTHFR* in TT homozygotes are  
5  
6 211 hypomethylated in both medial and lateral tissues, which seems to reflect a compensatory  
7  
8 212 higher expression of *MTHFR* gene. This is in line with a previous report of the complexity  
9  
10 213 of the effect of *MTHFR* variants on DNA methylation (De Gobbo, Price, Hanna, &  
11  
12 214 Robinson, 2018). A comprehensive metabolic assessment is necessary to advance our  
13  
14 215 understanding of one-carbon nutrients on DNA methylation involved in nsCL/P.

15  
16 216 The investigation of LINE-1 methylation in response to smoking does not necessarily  
17  
18 217 reflect changes in methylation at specific loci that have been reported to be influenced by  
19  
20 218 smoking (Andersen, Dogan, Beach, & Philibert, 2015). Hence, for understanding aspects  
21  
22 219 of the apparent mitigating effect of *mMTHFR* on smoking, we in future plan investigation  
23  
24 220 based on larger samples, and genetic loci/CpGs previously identified as being associated  
25  
26 221 with smoking and also implicated in non-syndromic OFCs (Joubert et al., 2016). Another  
27  
28 222 potential limitation of this study is non availability of RNA from these tissues to access  
29  
30 223 relationship between *mMTHFR* and its expression (mRNA level) - which can be  
31  
32 224 modulated by other epigenetic processes such as histone modification and micro-RNAs.  
33  
34 225 In this regard, there is evidence suggesting that miRNAs (miR-324-3p and miR-223), are  
35  
36 226 able to regulate *MTHFR* gene in salivary cells taken from nsCL/P cases (Grassia et al.,  
37  
38 227 2018). Once tissue collections still in process, are completed, we also plan to investigate  
39  
40 228 miRNAs and functional analysis in these tissues to better understand the complex  
41  
42 229 aetiology of nsCL/P.

43  
44 230 In conclusion, our study highlights the interplay of gene and environment in moderating  
45  
46 231 the establishment of methylation in medial and maxillary sides of the upper lip tissues.  
47  
48 232 The study requires replication in a larger study, including genes associated with smoking  
49  
50 233 and oral clefts. We consider that the study further champions the potential value of  
51  
52 234 investigating lip tissues, integrated with metabolomics for nutrient assessment, in order  
53  
54 235 to develop a clearer understanding of the aetio-pathogenesis of non-syndromic orofacial  
55  
56 236 clefts.

### 57 237 **Acknowledgements**

58  
59 238 The authors thank the patients and their families for their participation in the study. We  
60  
239 acknowledge the support received from the Department of Neuroscience and

1  
2  
3  
4 240 Rehabilitation, Section of Medical Biochemistry, Molecular Biology and Genetics,  
5  
6 241 University of Ferrara, Italy (n. 62040 & 0039804). Our sincere thanks to Dr. Houda  
7  
8 242 Oudouche, Department of Humanities, University of Ferrara, Italy and Lab. Group  
9  
10 243 members for their assistance. JL holds a Distinguished Research Chair in Chronic Disease  
11  
12 244 Epidemiology and Control.

## 14 245 **References**

- 16 246 Andersen, A. M., Dogan, M. V., Beach, S. R., & Philibert, R. A. (2015). Current and  
17  
18 247 Future Prospects for Epigenetic Biomarkers of Substance Use Disorders. *Genes*  
19  
20 248 (Basel), 6(4), 991-1022. doi:10.3390/genes6040991
- 22 249 Armstrong, R. A. (2014). When to use the Bonferroni correction. *Ophthalmic Physiol*  
23  
24 250 *Opt*, 34(5), 502-508. doi:10.1111/opo.12131
- 26 251 Asim, A., Agarwal, S., Panigrahi, I., Saiyed, N., & Bakshi, S. (2017). MTHFR promoter  
27  
28 252 hypermethylation may lead to congenital heart defects in Down syndrome.  
29  
30 253 *Intractable Rare Dis Res*, 6(4), 295-298. doi:10.5582/irdr.2017.01068
- 32 254 Beach, S. R. H., Lei, M. K., Ong, M. L., Brody, G. H., Dogan, M. V., & Philibert, R. A.  
33  
34 255 (2017). MTHFR methylation moderates the impact of smoking on DNA methylation  
35  
36 256 at AHRR for African American young adults. *Am J Med Genet B Neuropsychiatr*  
37  
38 257 *Genet*, 174(6), 608-618. doi:10.1002/ajmg.b.32544
- 40 258 Breton, C. V., Marsit, C. J., Faustman, E., Nadeau, K., Goodrich, J. M., Dolinoy, D. C.,  
41  
42 259 . . . Murphy, S. K. (2017). Small-Magnitude Effect Sizes in Epigenetic End Points  
43  
44 260 are Important in Children's Environmental Health Studies: The Children's  
45  
46 261 Environmental Health and Disease Prevention Research Center's Epigenetics  
47  
48 262 Working Group. *Environ Health Perspect*, 125(4), 511-526. doi:10.1289/EHP595
- 49 263 Coppede, F., Denaro, M., Tannorella, P., & Migliore, L. (2016). Increased MTHFR  
50  
51 264 promoter methylation in mothers of Down syndrome individuals. *Mutat Res*, 787, 1-  
52  
53 265 6. doi:10.1016/j.mrfmmm.2016.02.008
- 55 266 Coppede, F., Stoccoro, A., Tannorella, P., Gallo, R., Nicoli, V., & Migliore, L. (2019).  
56  
57 267 Association of Polymorphisms in Genes Involved in One-Carbon Metabolism with  
58  
59 268 MTHFR Methylation Levels. *Int J Mol Sci*, 20(15). doi:10.3390/ijms20153754

- 1  
2  
3  
4 269 Del Gobbo, G. F., Price, E. M., Hanna, C. W., & Robinson, W. P. (2018). No evidence  
5 270 for association of MTHFR 677C>T and 1298A>C variants with placental DNA  
6 271 methylation. *Clin Epigenetics*, 10, 34. doi:10.1186/s13148-018-0468-1  
7  
8  
9  
10 272 Dixon, M. J., Marazita, M. L., Beaty, T. H., & Murray, J. C. (2011). Cleft lip and palate:  
11 273 understanding genetic and environmental influences. *Nat Rev Genet*, 12(3), 167-178.  
12 274 doi:10.1038/nrg2933  
13  
14  
15  
16 275 Faul, F., Erdfelder, E., Lang, A. G., & Buchner, A. (2007). G\*Power 3: a flexible  
17 276 statistical power analysis program for the social, behavioral, and biomedical  
18 277 sciences. *Behav Res Methods*, 39(2), 175-191. doi:10.3758/bf03193146  
19  
20  
21  
22 278 Ge, J., Wang, J., Zhang, F., Diao, B., Song, Z. F., Shan, L. L., . . . Li, X. Q. (2015).  
23 279 Correlation between MTHFR gene methylation and pre-eclampsia, and its clinical  
24 280 significance. *Genet Mol Res*, 14(3), 8021-8028. doi:10.4238/2015.July.17.10  
25  
26  
27  
28 281 Grassia, V., Lombardi, A., Kawasaki, H., Ferri, C., Perillo, L., Mosca, L., . . . Caraglia,  
29 282 M. (2018). Salivary microRNAs as new molecular markers in cleft lip and palate.  
30 283 *Oncotarget*, 9(27), 18929-18938. doi:10.18632/oncotarget.24838  
31  
32  
33  
34 284 Jiang, R., Bush, J. O., & Lidral, A. C. (2006). Development of the upper lip:  
35 285 morphogenetic and molecular mechanisms. *Dev Dyn*, 235(5), 1152-1166.  
36 286 doi:10.1002/dvdy.20646  
37  
38  
39 287 Johnson, C. Y., & Little, J. (2008). Folate intake, markers of folate status and oral clefts:  
40 288 is the evidence converging? *Int J Epidemiol*, 37(5), 1041-1058.  
41 289 doi:10.1093/ije/dyn098  
42  
43  
44  
45 290 Joubert, B. R., Felix, J. F., Yousefi, P., Bakulski, K. M., Just, A. C., Breton, C., . . .  
46 291 London, S. J. (2016). DNA Methylation in Newborns and Maternal Smoking in  
47 292 Pregnancy: Genome-wide Consortium Meta-analysis. *Am J Hum Genet*, 98(4), 680-  
48 293 696. doi:10.1016/j.ajhg.2016.02.019  
49  
50  
51  
52  
53 294 Khan, M. F. J., Little, J., Mossey, P. A., Steegers-Theunissen, R. P. M., Bonsi, M., Bassi  
54 295 Andreasi, R., & Rubini, M. (2018a). Association between a common missense  
55 296 variant in LOXL3 gene and the risk of non-syndromic cleft palate. *Congenit Anom*  
56 297 (Kyoto), 58(4), 136-140. doi:10.1111/cga.12288  
57  
58  
59  
60

- 1  
2  
3  
4 298 Khan, M. F. J., Little, J., Mossey, P. A., Steegers-Theunissen, R. P., Autelitano, L.,  
5 299 Lombardo, I., . . . Rubini, M. (2018b). Evaluating LINE-1 methylation in cleft lip  
6 tissues and its association with early pregnancy exposures. *Epigenomics*, 10(1), 105-  
7 300 113. doi:10.2217/epi-2017-0081  
8 301  
9  
10  
11 302 Khan, M. F. J., Little, J., Abelli, L., Mossey, P. A., Autelitano, L., Nag, T. C., & Rubini,  
12 303 M. (2018c). Muscle fiber diameter assessment in cleft lip using image processing.  
13 *Oral Dis*, 24(3), 476-481. doi:10.1111/odi.12790  
14 304  
15  
16  
17 305 Khan, M. F. J., Little, J., Nag, T. C., Mossey, P. A., Autelitano, L., Meazzini, M. C., . .  
18 306 . Rubini, M. (2019a). Ultrastructural analysis of collagen fibril diameter distribution  
19 in cleft lip. *Oral Dis*, 25(1), 206-214. doi:10.1111/odi.12962  
20 307  
21  
22  
23 308 Khan, M. F. J., Little, J., Aleotti, V., Mossey, P. A., Steegers-Theunissen, R. P. M.,  
24 309 Autelitano, L., . . . Rubini, M. (2019b). LINE-1 methylation in cleft lip tissues:  
25 Influence of infant MTHFR c.677C>T genotype. *Oral Dis*. doi:10.1111/odi.13136  
26 310  
27  
28  
29 311 Leslie, E. J., & Marazita, M. L. (2013). Genetics of cleft lip and cleft palate. *Am J Med*  
30 312 *Genet C Semin Med Genet*, 163C(4), 246-258. doi:10.1002/ajmg.c.31381  
31  
32  
33 313 Liew, S. C., & Gupta, E. D. (2015). Methylenetetrahydrofolate reductase (MTHFR)  
34 C677T polymorphism: epidemiology, metabolism and the associated diseases. *Eur J*  
35 314 *Med Genet*, 58(1), 1-10. doi:10.1016/j.ejmg.2014.10.004  
36 315  
37  
38  
39 316 Lisanti, S., Omar, W. A., Tomaszewski, B., De Prins, S., Jacobs, G., Koppen, G., . . .  
40 317 Langie, S. A. (2013). Comparison of methods for quantification of global DNA  
41 methylation in human cells and tissues. *PLoS One*, 8(11), e79044.  
42 318 doi:10.1371/journal.pone.0079044  
43 319  
44  
45  
46 320 Little, J., Cardy, A., & Munger, R. G. (2004). Tobacco smoking and oral clefts: a meta-  
47 321 analysis. *Bull World Health Organ*, 82(3), 213-218.  
48  
49  
50  
51 322 Mandaviya, P. R., Joehanes, R., Aissi, D., Kuhnel, B., Marioni, R. E., Truong, V., . . .  
52 Consortium, B. (2017). Genetically defined elevated homocysteine levels do not  
53 323 result in widespread changes of DNA methylation in leukocytes. *PLoS One*, 12(10),  
54 324 e0182472. doi:10.1371/journal.pone.0182472  
55 325  
56  
57  
58  
59  
60

- 1  
2  
3  
4 326 McLeod, S. A. (2019, July 10). What does effect size tell you? Simply psychology:  
5  
6 327 Retrieved from <https://www.simplypsychology.org/effect-size.html>  
7
- 8 328 Mishra, J., Talwar, S., Kaur, L., Chandiok, K., Yadav, S., Puri, M., . . . Saraswathy, K.  
9  
10 329 N. (2019). Differential global and MTHFR gene specific methylation patterns in  
11  
12 330 preeclampsia and recurrent miscarriages: A case-control study from North India.  
13  
14 331 *Gene*, 704, 68-73. doi:10.1016/j.gene.2019.04.036  
15
- 16 332 Mossey, P. A., Shaw, W. C., Munger, R. G., Murray, J. C., Murthy, J., & Little, J.  
17  
18 333 (2011). Global oral health inequalities: challenges in the prevention and management  
19  
20 334 of orofacial clefts and potential solutions. *Adv Dent Res*, 23(2), 247-258.  
21  
22 335 doi:10.1177/0022034511402083  
23
- 24 336 Mossey, P. A., Little, J., Steegers-Theunissen, R., Molloy, A., Peterlin, B., Shaw, W.  
25  
26 337 C., . . . Rubini, M. (2017). Genetic Interactions in Nonsyndromic Orofacial Clefts in  
27  
28 338 Europe-EUROCRAN Study. *Cleft Palate Craniofac J*, 54(6), 623-630.  
29  
30 339 doi:10.1597/16-037  
31
- 32 340 Mossey, P. A., & Little, J. (2002). *Epidemiology of oral clefts: an international*  
33  
34 341 *perspective. In: Cleft Lip And Palate: From Origin To Treatment* (Wyszynski DF  
35  
36 342 Ed.). New York (NY): Oxford University Press.  
37
- 38 343 Nafee, T. M., Farrell, W. E., Carroll, W. D., Fryer, A. A., & Ismail, K. M. (2008).  
39  
40 344 Epigenetic control of fetal gene expression. *BJOG*, 115(2), 158-168.  
41  
42 345 doi:10.1111/j.1471-0528.2007.01528.x  
43
- 44 346 Nash, A. J., Mandaviya, P. R., Dib, M. J., Uitterlinden, A. G., van Meurs, J., Heil, S. G.,  
45  
46 347 . . . Ahmadi, K. R. (2019). Interaction between plasma homocysteine and the MTHFR  
48  
49 348 c.677C > T polymorphism is associated with site-specific changes in DNA  
50  
51 349 methylation in humans. *FASEB J*, 33(1), 833-843. doi:10.1096/fj.201800400R  
52
- 53 350 Ni, G., Qin, J., Chen, Z., Li, H., Zhou, J., Huang, M., & Zhou, L. (2018). Associations  
54  
55 351 between genetic variation in one-carbon metabolism and leukocyte DNA  
56  
57 352 methylation in valproate-treated patients with epilepsy. *Clin Nutr*, 37(1), 308-312.  
58  
59 353 doi:10.1016/j.clnu.2017.01.004  
60



- 1  
2  
3  
4 354 Pautz, N., Olivier, B., & Steyn, F. (2018). The use of parametric effect sizes in single  
5 study musculoskeletal physiotherapy research: A practical primer. *Phys Ther Sport*,  
6 355 32, 87-97. doi:10.1016/j.ptsp.2018.05.002  
7  
8 356  
9  
10 357 Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *BMJ*, 316(7139),  
11 358 1236-1238. doi:10.1136/bmj.316.7139.1236  
12  
13  
14 359 Piras, I. S., Costa, A., Tirindelli, M. C., Stoccoro, A., Huentelman, M. J., Sacco, R., . . .  
15 360 Lintas, C. (2020). Genetic and epigenetic MTHFR gene variants in the mothers of  
16 attention-deficit/hyperactivity disorder affected children as possible risk factors for  
17 361 neurodevelopmental disorders. *Epigenomics*. doi:10.2217/epi-2019-0356  
18  
19 362  
20  
21 363 Rai, V. (2018). Strong Association of C677T Polymorphism of  
22 Methylenetetrahydrofolate Reductase Gene With Nosyndromic Cleft Lip/Palate  
23 364 (nsCL/P). *Indian J Clin Biochem*, 33(1), 5-15. doi:10.1007/s12291-017-0673-2  
24  
25 365  
26  
27 366 Raut, J. R., Simeone, R. M., Tinker, S. C., Canfield, M. A., Day, R. S., & Agopian, A.  
28 367 J. (2019). Proportion of Orofacial Clefts Attributable to Recognized Risk Factors.  
29 *Cleft Palate Craniofac J*, 56(2), 151-158. doi:10.1177/1055665618774019  
30  
31 368  
32  
33 369 Reutter, H., Birnbaum, S., Lacava, A. D., Mende, M., Henschke, H., Berge, S., . . .  
34 370 Mangold, E. (2008). Family-based association study of the MTHFR polymorphism  
35 C677T in patients with nonsyndromic cleft lip and palate from central Europe. *Cleft*  
36 371 *Palate Craniofac J*, 45(3), 267-271. doi:10.1597/06-174  
37  
38 372  
39  
40 41 373 Richmond, R. C., & Joubert, B. R. (2017). Contrasting the effects of intra-uterine  
42 374 smoking and one-carbon micronutrient exposures on offspring DNA methylation.  
43 *Epigenomics*, 9(3), 351-367. doi:10.2217/epi-2016-0135  
44  
45 375  
46  
47 376 Shaw, G. M., Todoroff, K., Finnell, R. H., Rozen, R., & Lammer, E. J. (1999). Maternal  
48 377 vitamin use, infant C677T mutation in MTHFR, and isolated cleft palate risk. *Am J*  
49 *Med Genet*, 85(1), 84-85.  
50  
51 378  
52  
53 379 Sinclair, K. D., Allegrucci, C., Singh, R., Gardner, D. S., Sebastian, S., Bispham, J., . . .  
54 380 . Young, L. E. (2007). DNA methylation, insulin resistance, and blood pressure in  
55 offspring determined by maternal periconceptional B vitamin and methionine status.  
56 381 *Proc Natl Acad Sci U S A*, 104(49), 19351-19356. doi:10.1073/pnas.0707258104  
57  
58 382  
59  
60



- 1  
2  
3  
4 383 Stock, N. M., Humphries, K., Pourcain, B. S., Bailey, M., Persson, M., Ho, K. M., . . .  
5  
6 384 Sandy, J. (2016). Opportunities and Challenges in Establishing a Cohort Study: An  
7  
8 385 Example From Cleft Lip/Palate Research in the United Kingdom. *Cleft Palate*  
9  
10 386 *Craniofac J*, 53(3), 317-325. doi:10.1597/14-306
- 11  
12 387 Stover, P. J. (2009). One-carbon metabolism-genome interactions in folate-associated  
13  
14 388 pathologies. *J Nutr*, 139(12), 2402-2405. doi:10.3945/jn.109.113670
- 15  
16 389 van der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A., Trijbels, F. J., Eskes,  
17  
18 390 T. K., . . . Blom, H. J. (1998). A second common mutation in the  
19  
20 391 methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube  
21  
22 392 defects? *Am J Hum Genet*, 62(5), 1044-1051. doi:10.1086/301825
- 23  
24 393 Xuan, Z., Zhongpeng, Y., Yanjun, G., Jiaqi, D., Yuchi, Z., Bing, S., & Chenghao, L.  
25  
26 394 (2016). Maternal active smoking and risk of oral clefts: a meta-analysis. *Oral Surg*  
27  
28 395 *Oral Med Oral Pathol Oral Radiol*, 122(6), 680-690.  
29  
30 396 doi:10.1016/j.oooo.2016.08.007
- 31  
32 397 Zhou, Y., Sinnathamby, V., Yu, Y., Sikora, L., Johnson, C. Y., Mossey, P., & Little, J.  
33  
34 398 (2020). Folate intake, markers of folate status and oral clefts: An updated set of  
35  
36 399 systematic reviews and meta-analyses. *Birth Defects Res*, 112(19), 1699-1719.  
37  
38 400 doi:10.1002/bdr2.1827

Table 1. Mean methylation level (%) at LINE-1 and *MTHFR* gene promoter (m*MTHFR*) in medial and lateral cleft tissues.

Mean  $\pm$  standard deviation (SD) values of total non-syndromic CL/P cases or cases categorized by smoking and non-smoking, along with mean difference, 95% confidence interval (C.I) and nominal *p*-value of *t*-test.

<b>Infant DNA</b>	<b>Medial side</b> Mean $\pm$ SD	<b>Lateral side</b> Mean $\pm$ SD	<b>Mean difference</b> (95% C.I.)	<b><i>p</i>-value</b>
<b>LINE 1 (26)</b>				
<b>Total (n=26)</b>	<b>73.19 <math>\pm</math> 2.57</b>	<b>71.50 <math>\pm</math> 4.65</b>	1.69 (-0.64 to 4.02)	<i>p</i> =0.148
<b>Non-smoking</b> (n=15)	<b>73.78 <math>\pm</math> 2.48</b>	<b>70.22 <math>\pm</math> 4.95</b>	3.55 (0.51 to 6.59) <b><i>p</i>=0.025</b>	<i>p</i> =0.209
<b>Smoking (n=8)</b>	<b>72.00 <math>\pm</math> 2.56</b>	<b>72.38 <math>\pm</math> 5.15</b>	-0.38 (-5.70 to 4.94) <i>p</i> =0.872	
<b>m<i>MTHFR</i> (26)</b>				
<b>Total (n=26)</b>	<b>37.04 <math>\pm</math> 1.63</b>	<b>35.69 <math>\pm</math> 1.85</b>	1.34 (-6.69 to 9.38)	<i>p</i> =0.733
<b>Non-smoking</b> (n=15)	<b>37.50 <math>\pm</math> 1.82</b>	<b>38.28 <math>\pm</math> 1.81</b>	-0.77 (-10.33 to 8.77) <i>p</i> =0.866	<i>p</i> =0.554
<b>Smoking (n=8)</b>	<b>31.13 <math>\pm</math> 11.50</b>	<b>25.00 <math>\pm</math> 11.73</b>	6.12 (-6.60 to 18.85) <i>p</i> =0.293	

Abbreviations: n, number of cases; SD, standard deviation; CI, confidence interval.

Footnote: Maternal smoking data was available for only 23 cases.

Table 2. *mMTHFR* level (%) in medial and lateral cleft lip tissues of total non-syndromic CL/P cases, stratified by *MTHFR* c.677C>T genotype.

<b>MTHFR c.677C&gt;T</b>	<b>Medial side Mean ± SD</b>	<b>Lateral side Mean ± SD</b>	<b>Mean difference (95% C.I.)</b>	<b><i>p</i>-value*</b>
<b><i>mMTHFR</i> (26)</b>				
CC (n=7)	<b>44.57 ± 1.46</b>	<b>37.71 ± 2.11</b>	6.86 (-17.57 to 31.28)	ref.
CT (n=13)	<b>38.54 ± 16.62</b>	<b>37.77 ± 18.90</b>	0.7 (-9.97 to 11.51)	<i>p</i> =0.649
TT (n=6)	<b>25.00 ± 12.36</b>	<b>28.83 ± 15.94</b>	-3.83 (-22.28 to 14.61)	<i>p</i> =0.390

Abbreviations: n, number of cases; SD, standard deviation; CI, confidence interval; ref., reference.

\*Nominal *p*-value of comparisons of mean difference between medial and lateral sides considering CC genotype as reference.

1  
2  
3  
4 **1** *MTHFR* promoter methylation might mitigate the effect of smoking at the level of  
5  
6 **2** *LINE-1* in cleft lip tissues – a preliminary study  
7

8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

3 Mohammad Faisal J Khan, Julian Little, Peter A Mossey, Azeez Butali, Luca Autelitano,  
4 Maria C Meazzini, Michele Rubini

5 **Mohammad Faisal J. Khan:** Department of Neuroscience and Rehabilitation, Section  
6 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara,  
7 Ferrara, Italy. Email: khnmmm@unife.it

8 **Julian Little:** School of Epidemiology and Public Health, University of Ottawa, Ottawa,  
9 Ontario, Canada. Email: jlittle@uottawa.ca

10 **Peter A. Mossey:** Craniofacial Development at the World Health Organization–  
11 collaborating Centre for Oral and Craniofacial Research, Dental Hospital and School,  
12 University of Dundee, Dundee, Scotland. Email: p.a.mossey@dundee.ac.uk

13 **Azeez Butali:** Department of Oral Pathology, Radiology and Medicine, College of  
14 Dentistry, University of Iowa, Iowa City, IA, USA. Email: azeez-butali@uiowa.edu

15 **Luca Autelitano:** Smile House Milan, Regional Centre for Orofacial Clefts and  
16 Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo  
17 Hospital, University of Milan, Milan, Italy. Email: luca.aute@gmail.com

**Maria C Meazzini:** Smile House Milan, Regional Centre for Orofacial Clefts and  
Craniofacial Anomalies, Department of Cranio-Maxillo-Facial Surgery, San Paolo  
Hospital, University of Milan, Milan, Italy. Email: cmeazzini@yahoo.it

18 **Michele Rubini:** Department of Neuroscience and Rehabilitation, Section of Medical  
19 Biochemistry, Molecular Biology and Genetics, University of Ferrara, Ferrara, Italy.  
20 Email: rub@unife.it

21  
22 **Corresponding author.**

23 **Mohammad Faisal J. Khan,** Department of Neuroscience and Rehabilitation, Section  
24 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Via  
25 Fossato di Mortara 74 I-44121, Ferrara, Italy. Email: khnmmm@unife.it

26  
27 **Funding information.** This study was partly supported by FAR-2018 grant from University  
28 of Ferrara and from Department of Biomedical and Specialty Surgical Sciences, Section  
29 of Medical Biochemistry, Molecular Biology and Genetics, University of Ferrara, Italy,  
30 Grant/Award Number: 62040 and 0039804

1  
2  
3  
4 **Abstract**

5  
6 **Background:** The medial and maxillary aspects of the upper lip originate at separate  
7 embryonic stages and therefore may experience different maternal exposure patterns  
8 which may affect methylation. Based on this hypothesis, we investigated the level of  
9 methylation of the methylene tetrahydrofolate reductase promoter gene (*mMTHFR*) in  
10 tissues from cleft lip, and *mMTHFR* levels by *MTHFR* c.677C>T genotype. We further  
11 investigated whether *mMTHFR* mitigates the effect of smoking on long interspersed  
12 nuclear element (LINE-1) methylation in these tissues.  
13  
14

15  
16 **Methods.** DNA extracted from medial and lateral tissues of 26 infants with non-  
17 syndromic cleft lip with or without cleft palate (nsCL/P) was bisulfite converted and  
18 *mMTHFR* was measured on a pyrosequencer. LINE-1 methylation and *MTHFR*  
19 c.677C>T genotype data were obtained in our previous study.  
20  
21

22  
23 **Results.** There was no substantial difference in *mMTHFR* ( $p=0.733$ ) and LINE-1  
24 ( $p=0.148$ ) between the two tissues. *mMTHFR* was not influenced by *MTHFR* c.677C>T  
25 genotype, but there was suggestive evidence that the difference was larger among infants  
26 exposed to maternal smoking compared to non-exposed. LINE-1 methylation differences  
27 were significant ( $p=0.025$ ) in infants born to non-smoking mothers, but this was not  
28 apparent ( $p=0.872$ ) in infants born to mothers who smoked. Our Pearson's correlation  
29 analysis suggested a weak inverse association between *mMTHFR* and LINE-1 ( $r=-0.179$ ;  
30  $p=0.381$ ).  
31  
32

33  
34 **Conclusion.** Our preliminary observation of differences in patterns of *mMTHFR* levels  
35 in lip tissue suggests the interplay of gene and environment in establishment of  
36 methylation in tissues at both sides of cleft lip. This requires investigation in a larger  
37 cohort, integrated with metabolic assessment.  
38  
39

40  
41 **Keywords:** Non-syndromic cleft lip with or without cleft palate, DNA methylation,  
42 *MTHFR* c.677C>T, LINE-1, *MTHFR* promoter methylation  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## 59 Introduction

60 Orofacial clefts (OFCs) are collectively among the most common human congenital  
61 anomalies that can occur in isolation or as part of a syndrome (Mossey et al., 2011). Some  
62 environmental and multiple genetic risk factors have been identified for non-syndromic  
63 form of OFCs (Leisle & Marazita, 2013; Khan et al., 2018a, Mossey et al., 2017; Raut et  
64 al., 2019; Johnson & Little, 2008; Little, Cardy, & Munger, 2004) but the causes of these  
65 defects remain largely unknown.

66 OFCs develop in early life, when the embryo is extremely susceptible to perturbation of  
67 the in-utero environment (Dixon, Marazita, & Beaty, 2010). Among environmental  
68 factors, tobacco smoking has been found to influence facial morphology (Xuan et al.,  
69 2016), and is reported to be the most consistent and strongest risk factor for OFCs (Raut  
70 et al., 2019). Such perturbation of the early life environment affects developmental  
71 programming in the embryo, with sustained changes potentially detectable in tissues from  
72 medial nasal and maxillary sides of the upper lip in cases with non-syndromic cleft lip  
73 with or without cleft palate (nsCL/P) as observed by our group in recent studies (Khan et  
74 al., 2018b, Khan et al., 2018c, Khan et al., 2019a, Khan et al., 2019b).

75 Methylene tetrahydrofolate reductase (MTHFR) is the rate-limiting enzyme in the one-  
76 carbon cycle, a pathway that is critical to metabolism of folate. Folate is a specific nutrient  
77 involved in development of craniofacial structures (Jiang, Bush, & Lidral, 2006) and  
78 provides methyl group for DNA methylation (Sinclair et al., 2007). MTHFR activity is  
79 mainly regulated by the combination of two mechanistic aspects – 1) variants within the  
80 gene that essentially acts at the level of enzyme activity and specificity; 2) methylation  
81 of the gene promoter that mainly affects level of expression. Both the c.677C>T  
82 (rs1801133) and c.1298A>C (rs1801131) variants of the *MTHFR* gene have been  
83 demonstrated to reduce enzyme activity (Liew & Gupta, 2015; van der Put et al., 1998).  
84 The effect of both these variants have been investigated in relation to nsCL/P in the index  
85 child, and/or one or both parents but the results from these studies have been inconclusive  
86 (Rai, 2018; Mossey, & Little, 2002; Reutter et al., 2008; Shaw, Todoroff, Finnell, Rozen,  
87 & Lammer, 1999; Zhou et al., 2020). Our group; however, considered an alternative  
88 mechanism involving DNA methylation to decipher the role of *MTHFR* gene variants in

89 nsCL/P, and found that a variant in *MTHFR* gene plays a role in the establishment of  
90 methylation in cleft lip tissues (Khan et al., 2019b).

91 We know that methylation within *MTHFR* promoter (*mMTHFR*) contributes to variation  
92 in *MTHFR* protein activity similar to that conferred by *MTHFR* variants (Coppede,  
93 Denaro, Tannorella, & Migliore, 2016), and has been shown to contribute to many  
94 developmental (Asim, Agarwal, Panigrahi, Sai, yed, & Bakshi, 2017; Coppede et al.,  
95 2016) and pregnancy related disorders (Ge et al., 2015; Mishra et al., 2019). However, to  
96 our knowledge, there appears to be no evidence of information regarding the methylation  
97 profile of *mMTHFR*, or the contribution of *MTHFR* variants to *MTHFR* methylation level  
98 in nsCL/P. Therefore, we undertook this preliminary study to assess the level of  
99 *mMTHFR*, and further investigate the relationship between *MTHFR* c.677C>T variant  
100 and *mMTHFR* utilizing tissues from medial and lateral aspects of the upper lip in  
101 individuals with nsCL/P.

102 *mMTHFR* could also be involved in differences in regulation of methylation repair  
103 activity and hence might contribute to individual differences by altering enzyme activity.  
104 This could either affect the availability of activated methyl group or increase the rate of  
105 loss of methylation (over time) in response to exposures associated with demethylation  
106 such as cigarette smoking (Beach et al., 2017). Alternatively, when *MTHFR* is more  
107 active, the availability of methyl group is more likely enhanced, potentially alleviating  
108 the impact of exposures such as smoking that would otherwise cause demethylation  
109 (Beach et al., 2017; Stover, 2009). Intrigued by this concept, we examined whether  
110 *mMTHFR* in the indexed infant could mitigate the adverse effects of active maternal  
111 smoking exposures, and potentially be reflected as changes in LINE-1 methylation level  
112 – widely accepted to be a proxy for overall genomic DNA methylation content (Lisanti  
113 et al., 2013).

## 114 **Materials and Methods**

115 Tissue samples from the medial and lateral sides of cleft lip were collected from 26 cases  
116 with nsCL/P that were recruited between 2016 and 2018 in the Centre for Orofacial Clefts  
117 and Craniofacial Anomalies, San Paolo Hospital, Milan, Italy (PENTACLEFT: prot. no.  
118 08–2011). Our sample included 13 female and 13 male cases. Fifteen cases had cleft lip



1  
2  
3  
4 119 and 10 had cleft lip and palate (phenotypic data missing for one case – due to  
5 120 mismatching). Among the mothers of these 26 cases, fifteen were non-smokers and eight  
6 121 actively smoked during the periconceptional period – 3 months before to 3 months after  
7 122 conception (smoking data missing for three mothers – due to non-response on survey).

11  
12 123 The DNA extracted from tissues were bisulfite converted and methylation of the *MTHFR*  
13 124 gene promoter was measured using PyroMark Q96 predesigned CpG assay  
14 125 (#PM00000091) on a PyroMark Q96 ID pyrosequencer (Qiagen GmbH, Hilden,  
15 126 Germany), with minor modification of the method previously described (Khan et al.,  
16 127 2018b). Briefly, the amplification of bisulfite converted DNA was performed by PCR  
17 128 with *MTHFR* primer, and pyrosequencing done using *MTHFR* specific sequencing  
18 129 primers. The predesigned assay contained the following sequence 5'-  
19 130 GGTCAGTACCGATGGGGGCGAGGAYACGGGC-3' (prior to bisulfite  
20 131 conversion) including 3 CpG sites to assess in promoter region of *MTHFR*. The  
21 132 nucleotide dispensation order was:  
22 133 TGTCATGATGATATCGAGTGGTCGAGATATCG. LINE-1 methylation and  
23 134 *MTHFR* c.677C>T genotype data for this cohort were obtained in our previous study  
24 135 (Khan et al., 2019b). The Kolmogorov-Smirnov test showed that the data were normally  
25 136 distributed, hence, parametric comparison of within and between samples were performed  
26 137 using Student's *t*-test. In addition, we report parametric effect size estimate (Hedge's  $g_s$ )  
27 138 associated with independent sample Student's *t*-test, and Pearson's correlation to  
28 139 determine relationship between m*MTHFR* and LINE-1 (Pautz, Olivier, & Steyn, 2018;  
29 140 McLeod, 2019). Considering the total of 26 cases included in this study, we calculated  
30 141 statistical power using G\*POWER software 3.1.9.2 version (Faul, Erdfelder, Lang, &  
31 142 Buchner, 2007). Considering a two-tailed *t*-test and an equally serious  $\alpha$  &  $\beta$  error ( $\beta/\alpha =$   
32 143 1), an effect size  $d_z$  of 0.2, we calculated the power (1- $\beta$ -error) of 0.60.

## 33 144 **Results**

34 145 We found a non-significant difference of 1.3% in m*MTHFR* between medial and lateral  
35 146 tissues ( $p=0.733$ ; Table 1). m*MTHFR* was not influenced by *MTHFR* c.677C>T genotype  
36 147 (Table 2). Similarly, LINE-1 methylation was not significantly different (1.7%) across  
37 148 tissues in this cohort ( $p=0.148$ ; Table 1).

1  
2  
3  
4 149 To examine the role of *mMTHFR* as a source in compensating for the effect of smoking  
5  
6 150 on LINE-1 methylation level, we compared the level of methylation in *MTHFR* and  
7  
8 151 LINE-1 in medial and lateral tissues between infants born to mothers who smoked in the  
9  
10 152 periconceptual period and infants of non-smoking mothers. Among infants exposed to  
11  
12 153 smoking, the difference in *mMTHFR* was larger (6.1%) but showed a lowered level of  
13  
14 154 methylation ( $p=0.293$ ; Table 1) compared to infants born to non-smoking mothers in  
15  
16 155 whom *mMTHFR* was similar ( $\sim 38\%$ ) in the two tissues ( $p=0.866$ ; Table 1). Interestingly,  
17  
18 156 LINE-1 methylation differences were significant ( $p=0.025$ ) in infants born to non-  
19  
20 157 smoking mothers; this however, was not apparent ( $p=0.872$ ) in infants born to mothers  
21  
22 158 who smoked, with medial and lateral tissues showing equal level (72%) of methylation  
23  
24 159 (Table 1).

25  
26 160 Comparisons between these groups were non-significant for both *mMTHFR* ( $p=0.554$ )  
27  
28 161 and LINE-1 ( $p=0.209$ ). We also calculated the effect size (ES) for between comparisons  
29  
30 162 and found a small ES for *mMTHFR* ( $gs=0.26$ ). While LINE-1 showed medium ( $gs=0.56$ )  
31  
32 163 ES, our Pearson's correlation analysis suggested a weak inverse association between  
33  
34 164 *mMTHFR* and LINE-1 ( $r= -0.179$ ;  $p=0.381$ ).

## 35 36 37 165 **Discussion**

38  
39 166 In this preliminary study, we found a small non-significant difference in *mMTHFR* and  
40  
41 167 LINE-1 methylation across medial and lateral tissues. A difference in *mMTHFR* was  
42  
43 168 observed in infants of mothers who smoked but not among infants born to non-smoking  
44  
45 169 mothers. By contrast, a significant difference in LINE-1 methylation was apparent in  
46  
47 170 infants born to non-smoking mothers, but not in infants born to smoking mothers.

48  
49 171 Our observation of small changes in methylation in *mMTHFR* and LINE-1 is compatible  
50  
51 172 with reports that the magnitude of epigenetic effect associated with exposure in children  
52  
53 173 is generally small; large changes may not be compatible with continued development  
54  
55 174 (Breton et al., 2017). Hence, a small imbalance in methylation in progeny cells of the  
56  
57 175 medial and maxillary tissues might result in an apparently small distinction between  
58  
59 176 sufficient and insufficient methylation. Insufficient methylation might in turn interfere  
60  
177 with the fusion process, so leading to the development of a cleft lip. Persistence of this  
178 small imbalance throughout pregnancy and into the postnatal period would be manifested

1  
2  
3  
4 179 as differences in tissues from medial and lateral side of cleft lip. Such a difference could  
5  
6 180 therefore provide insight into epigenetic effects of early life environmental exposures  
7  
8 181 (Richmond et al., 2017).  
9

10 182 The pattern of *mMTHFR* levels suggests that nearly equal levels of folate are available  
11 183 for tissues developing at distinct embryonic periods, but the level of availability could  
12 184 likely be influenced by external factors such as smoking (Nafee, Farrell, Carroll, Fryer,  
13 185 & Ismail, 2008). Importantly, we found lower *mMTHFR* in the smoking group that  
14 186 suggests an increase in folate availability. This increased folate availability might provide  
15 187 methyl-group to mitigate/overcome the effect of smoking resulting in the observation of  
16 188 nearly equal levels of LINE-1 methylation in the medial and lateral tissues (72%). The  
17 189 lower *mMTHFR* level in the smoking group further suggests smoking-associated  
18 190 demethylation at a single gene promoter, whereas there was little difference in global  
19 191 methylation. This is in line with the suggestion that small changes in global methylation  
20 192 of developing tissues might have substantial effects in the longer term (Breton et al.,  
21 193 2017). Pearson's correlation analysis showed a weak association between *mMTHFR* and  
22 194 LINE-1. This provides some support for a role of *mMTHFR* in moderating epigenetic  
23 195 response to smoking, and our previous findings that lip tissues are highly responsive to  
24 196 maternal environmental exposures (Khan et al., 2018b). We acknowledge that our results  
25 197 are based on small sample size, because collecting tissues from the cleft cases presents  
26 198 considerable challenges (Stock et al., 2016). We did not correct for multiple comparisons  
27 199 because reducing the risk of type I error can be at the expense of increasing type II error,  
28 200 and because of the preliminary nature of the study, identifying hypotheses for further  
29 201 investigation (Perneger 1998; Armstrong, 2014). A limitation of using tissue from the  
30 202 clefts is the difficulty of obtaining an appropriate reference group from which lip tissue  
31 203 samples could be collected. This problem arises from concerns about ethical issues and  
32 204 selection bias and is highly likely to be encountered in other studies.  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50

51 205 Our result is consistent with previous reports involving a large number of healthy Italian  
52 206 participants showing no association of *MTHFR* c.677C>T with *mMTHFR* indicating that  
53 207 c.677C>T variant does not act as a *cis* regulatory element to regulate its own gene  
54 208 promoter (Piras et al., 2020; Coppede et al., 2019; Ni et al., 2017), although there are  
55 209 reports that *MTHFR* c.677C>T genotype influences *mMTHFR* (Mandaviya et al. 2017;  
56  
57  
58  
59  
60

1  
2  
3  
4 210 Nash et al., 2019). Accordingly, we found that *mMTHFR* in TT homozygotes are  
5  
6 211 hypomethylated in both medial and lateral tissues, which seems to reflect a compensatory  
7  
8 212 higher expression of *MTHFR* gene. This is in line with a previous report of the complexity  
9  
10 213 of the effect of *MTHFR* variants on DNA methylation (De Gobbo, Price, Hanna, &  
11  
12 214 Robinson, 2018). A comprehensive metabolic assessment is necessary to advance our  
13  
14 215 understanding of one-carbon nutrients on DNA methylation involved in nsCL/P.

15  
16 216 The investigation of LINE-1 methylation in response to smoking does not necessarily  
17  
18 217 reflect changes in methylation at specific loci that have been reported to be influenced by  
19  
20 218 smoking (Andersen, Dogan, Beach, & Philibert, 2015). Hence, for understanding aspects  
21  
22 219 of the apparent mitigating effect of *mMTHFR* on smoking, we in future plan investigation  
23  
24 220 based on larger samples, and genetic loci/CpGs previously identified as being associated  
25  
26 221 with smoking and also implicated in non-syndromic OFCs (Joubert et al., 2016). Another  
27  
28 222 potential limitation of this study is non availability of RNA from these tissues to access  
29  
30 223 relationship between *mMTHFR* and its expression (mRNA level) - which can be  
31  
32 224 modulated by other epigenetic processes such as histone modification and micro-RNAs.  
33  
34 225 In this regard, there is evidence suggesting that miRNAs (miR-324-3p and miR-223), are  
35  
36 226 able to regulate *MTHFR* gene in salivary cells taken from nsCL/P cases (Grassia et al.,  
37  
38 227 2018). Once tissue collections still in process, are completed, we also plan to investigate  
39  
40 228 miRNAs and functional analysis in these tissues to better understand the complex  
41  
42 229 aetiology of nsCL/P.

43  
44 230 In conclusion, our study highlights the interplay of gene and environment in moderating  
45  
46 231 the establishment of methylation in medial and maxillary sides of the upper lip tissues.  
47  
48 232 The study requires replication in a larger study, including genes associated with smoking  
49  
50 233 and oral clefts. We consider that the study further champions the potential value of  
51  
52 234 investigating lip tissues, integrated with metabolomics for nutrient assessment, in order  
53  
54 235 to develop a clearer understanding of the aetio-pathogenesis of non-syndromic orofacial  
55  
56 236 clefts.

### 57 237 **Acknowledgements**

58  
59 238 The authors thank the patients and their families for their participation in the study. We  
60  
239 acknowledge the support received from the Department of Neuroscience and

1  
2  
3  
4 240 Rehabilitation, Section of Medical Biochemistry, Molecular Biology and Genetics,  
5  
6 241 University of Ferrara, Italy (n. 62040 & 0039804). Our sincere thanks to Dr. Houda  
7  
8 242 Oudouche, Department of Humanities, University of Ferrara, Italy and Lab. Group  
9  
10 243 members for their assistance. JL holds a Distinguished Research Chair in Chronic Disease  
11  
12 244 Epidemiology and Control.

## 14 245 **References**

16 246 Andersen, A. M., Dogan, M. V., Beach, S. R., & Philibert, R. A. (2015). Current and  
17  
18 247 Future Prospects for Epigenetic Biomarkers of Substance Use Disorders. *Genes*  
19  
20 248 (Basel), 6(4), 991-1022. doi:10.3390/genes6040991

22 249 **Armstrong, R. A. (2014). When to use the Bonferroni correction. *Ophthalmic Physiol***  
23  
24 250 ***Opt*, 34(5), 502-508. doi:10.1111/opo.12131**

26 251 Asim, A., Agarwal, S., Panigrahi, I., Saiyed, N., & Bakshi, S. (2017). MTHFR promoter  
27  
28 252 hypermethylation may lead to congenital heart defects in Down syndrome.  
29  
30 253 *Intractable Rare Dis Res*, 6(4), 295-298. doi:10.5582/irdr.2017.01068

32 254 Beach, S. R. H., Lei, M. K., Ong, M. L., Brody, G. H., Dogan, M. V., & Philibert, R. A.  
33  
34 255 (2017). MTHFR methylation moderates the impact of smoking on DNA methylation  
35  
36 256 at AHRR for African American young adults. *Am J Med Genet B Neuropsychiatr*  
37  
38 257 *Genet*, 174(6), 608-618. doi:10.1002/ajmg.b.32544

40 258 Breton, C. V., Marsit, C. J., Faustman, E., Nadeau, K., Goodrich, J. M., Dolinoy, D. C.,  
41  
42 259 . . . Murphy, S. K. (2017). Small-Magnitude Effect Sizes in Epigenetic End Points  
43  
44 260 are Important in Children's Environmental Health Studies: The Children's  
45  
46 261 Environmental Health and Disease Prevention Research Center's Epigenetics  
47  
48 262 Working Group. *Environ Health Perspect*, 125(4), 511-526. doi:10.1289/EHP595

49 263 Coppede, F., Denaro, M., Tannorella, P., & Migliore, L. (2016). Increased MTHFR  
50  
51 264 promoter methylation in mothers of Down syndrome individuals. *Mutat Res*, 787, 1-  
52  
53 265 6. doi:10.1016/j.mrfmmm.2016.02.008

55 266 Coppede, F., Stoccoro, A., Tannorella, P., Gallo, R., Nicoli, V., & Migliore, L. (2019).  
56  
57 267 Association of Polymorphisms in Genes Involved in One-Carbon Metabolism with  
58  
59 268 MTHFR Methylation Levels. *Int J Mol Sci*, 20(15). doi:10.3390/ijms20153754

- 1  
2  
3  
4 269 Del Gobbo, G. F., Price, E. M., Hanna, C. W., & Robinson, W. P. (2018). No evidence  
5 270 for association of MTHFR 677C>T and 1298A>C variants with placental DNA  
6 271 methylation. *Clin Epigenetics*, 10, 34. doi:10.1186/s13148-018-0468-1  
7  
8  
9  
10 272 Dixon, M. J., Marazita, M. L., Beaty, T. H., & Murray, J. C. (2011). Cleft lip and palate:  
11 273 understanding genetic and environmental influences. *Nat Rev Genet*, 12(3), 167-178.  
12 274 doi:10.1038/nrg2933  
13  
14  
15  
16 275 Faul, F., Erdfelder, E., Lang, A. G., & Buchner, A. (2007). G\*Power 3: a flexible  
17 276 statistical power analysis program for the social, behavioral, and biomedical  
18 277 sciences. *Behav Res Methods*, 39(2), 175-191. doi:10.3758/bf03193146  
19  
20  
21  
22 278 Ge, J., Wang, J., Zhang, F., Diao, B., Song, Z. F., Shan, L. L., . . . Li, X. Q. (2015).  
23 279 Correlation between MTHFR gene methylation and pre-eclampsia, and its clinical  
24 280 significance. *Genet Mol Res*, 14(3), 8021-8028. doi:10.4238/2015.July.17.10  
25  
26  
27  
28 281 Grassia, V., Lombardi, A., Kawasaki, H., Ferri, C., Perillo, L., Mosca, L., . . . Caraglia,  
29 282 M. (2018). Salivary microRNAs as new molecular markers in cleft lip and palate.  
30 283 *Oncotarget*, 9(27), 18929-18938. doi:10.18632/oncotarget.24838  
31  
32  
33  
34 284 Jiang, R., Bush, J. O., & Lidral, A. C. (2006). Development of the upper lip:  
35 285 morphogenetic and molecular mechanisms. *Dev Dyn*, 235(5), 1152-1166.  
36 286 doi:10.1002/dvdy.20646  
37  
38  
39 287 Johnson, C. Y., & Little, J. (2008). Folate intake, markers of folate status and oral clefts:  
40 288 is the evidence converging? *Int J Epidemiol*, 37(5), 1041-1058.  
41 289 doi:10.1093/ije/dyn098  
42  
43  
44  
45 290 Joubert, B. R., Felix, J. F., Yousefi, P., Bakulski, K. M., Just, A. C., Breton, C., . . .  
46 291 London, S. J. (2016). DNA Methylation in Newborns and Maternal Smoking in  
47 292 Pregnancy: Genome-wide Consortium Meta-analysis. *Am J Hum Genet*, 98(4), 680-  
48 293 696. doi:10.1016/j.ajhg.2016.02.019  
49  
50  
51  
52  
53 294 Khan, M. F. J., Little, J., Mossey, P. A., Steegers-Theunissen, R. P. M., Bonsi, M., Bassi  
54 295 Andreasi, R., & Rubini, M. (2018a). Association between a common missense  
55 296 variant in LOXL3 gene and the risk of non-syndromic cleft palate. *Congenit Anom*  
56 297 (Kyoto), 58(4), 136-140. doi:10.1111/cga.12288  
57  
58  
59  
60



- 1  
2  
3  
4 298 Khan, M. F. J., Little, J., Mossey, P. A., Steegers-Theunissen, R. P., Autelitano, L.,  
5 299 Lombardo, I., . . . Rubini, M. (2018b). Evaluating LINE-1 methylation in cleft lip  
6 tissues and its association with early pregnancy exposures. *Epigenomics*, 10(1), 105-  
7 300 113. doi:10.2217/epi-2017-0081  
8 301
- 11 302 Khan, M. F. J., Little, J., Abelli, L., Mossey, P. A., Autelitano, L., Nag, T. C., & Rubini,  
12 303 M. (2018c). Muscle fiber diameter assessment in cleft lip using image processing.  
13 304 *Oral Dis*, 24(3), 476-481. doi:10.1111/odi.12790
- 17 305 Khan, M. F. J., Little, J., Nag, T. C., Mossey, P. A., Autelitano, L., Meazzini, M. C., . . .  
18 306 . Rubini, M. (2019a). Ultrastructural analysis of collagen fibril diameter distribution  
19 307 in cleft lip. *Oral Dis*, 25(1), 206-214. doi:10.1111/odi.12962
- 23 308 Khan, M. F. J., Little, J., Aleotti, V., Mossey, P. A., Steegers-Theunissen, R. P. M.,  
24 309 Autelitano, L., . . . Rubini, M. (2019b). LINE-1 methylation in cleft lip tissues:  
25 310 Influence of infant MTHFR c.677C>T genotype. *Oral Dis*. doi:10.1111/odi.13136
- 29 311 Leslie, E. J., & Marazita, M. L. (2013). Genetics of cleft lip and cleft palate. *Am J Med*  
30 312 *Genet C Semin Med Genet*, 163C(4), 246-258. doi:10.1002/ajmg.c.31381
- 33 313 Liew, S. C., & Gupta, E. D. (2015). Methylenetetrahydrofolate reductase (MTHFR)  
34 314 C677T polymorphism: epidemiology, metabolism and the associated diseases. *Eur J*  
35 315 *Med Genet*, 58(1), 1-10. doi:10.1016/j.ejmg.2014.10.004
- 39 316 Lisanti, S., Omar, W. A., Tomaszewski, B., De Prins, S., Jacobs, G., Koppen, G., . . .  
40 317 Langie, S. A. (2013). Comparison of methods for quantification of global DNA  
41 318 methylation in human cells and tissues. *PLoS One*, 8(11), e79044.  
42 319 doi:10.1371/journal.pone.0079044
- 46 320 Little, J., Cardy, A., & Munger, R. G. (2004). Tobacco smoking and oral clefts: a meta-  
47 321 analysis. *Bull World Health Organ*, 82(3), 213-218.
- 51 322 Mandaviya, P. R., Joehanes, R., Aissi, D., Kuhnel, B., Marioni, R. E., Truong, V., . . .  
52 323 Consortium, B. (2017). Genetically defined elevated homocysteine levels do not  
53 324 result in widespread changes of DNA methylation in leukocytes. *PLoS One*, 12(10),  
54 325 e0182472. doi:10.1371/journal.pone.0182472



- 1  
2  
3  
4 326 McLeod, S. A. (2019, July 10). What does effect size tell you? Simply psychology:  
5  
6 327 Retrieved from <https://www.simplypsychology.org/effect-size.html>  
7
- 8 328 Mishra, J., Talwar, S., Kaur, L., Chandiok, K., Yadav, S., Puri, M., . . . Saraswathy, K.  
9  
10 329 N. (2019). Differential global and MTHFR gene specific methylation patterns in  
11  
12 330 preeclampsia and recurrent miscarriages: A case-control study from North India.  
13  
14 331 *Gene*, 704, 68-73. doi:10.1016/j.gene.2019.04.036  
15
- 16 332 Mossey, P. A., Shaw, W. C., Munger, R. G., Murray, J. C., Murthy, J., & Little, J.  
17  
18 333 (2011). Global oral health inequalities: challenges in the prevention and management  
19  
20 334 of orofacial clefts and potential solutions. *Adv Dent Res*, 23(2), 247-258.  
21  
22 335 doi:10.1177/0022034511402083  
23
- 24 336 Mossey, P. A., Little, J., Steegers-Theunissen, R., Molloy, A., Peterlin, B., Shaw, W.  
25  
26 337 C., . . . Rubini, M. (2017). Genetic Interactions in Nonsyndromic Orofacial Clefts in  
27  
28 338 Europe-EUROCRAN Study. *Cleft Palate Craniofac J*, 54(6), 623-630.  
29  
30 339 doi:10.1597/16-037  
31
- 32 340 Mossey, P. A., & Little, J. (2002). *Epidemiology of oral clefts: an international*  
33  
34 341 *perspective. In: Cleft Lip And Palate: From Origin To Treatment* (Wyszynski DF  
35  
36 342 Ed.). New York (NY): Oxford University Press.  
37
- 38 343 Nafee, T. M., Farrell, W. E., Carroll, W. D., Fryer, A. A., & Ismail, K. M. (2008).  
39  
40 344 Epigenetic control of fetal gene expression. *BJOG*, 115(2), 158-168.  
41  
42 345 doi:10.1111/j.1471-0528.2007.01528.x  
43
- 44 346 Nash, A. J., Mandaviya, P. R., Dib, M. J., Uitterlinden, A. G., van Meurs, J., Heil, S. G.,  
45  
46 347 . . . Ahmadi, K. R. (2019). Interaction between plasma homocysteine and the MTHFR  
48  
49 348 c.677C > T polymorphism is associated with site-specific changes in DNA  
50  
51 349 methylation in humans. *FASEB J*, 33(1), 833-843. doi:10.1096/fj.201800400R  
52
- 53 350 Ni, G., Qin, J., Chen, Z., Li, H., Zhou, J., Huang, M., & Zhou, L. (2018). Associations  
54  
55 351 between genetic variation in one-carbon metabolism and leukocyte DNA  
56  
57 352 methylation in valproate-treated patients with epilepsy. *Clin Nutr*, 37(1), 308-312.  
58  
59 353 doi:10.1016/j.clnu.2017.01.004  
60

- 1  
2  
3  
4 354 Pautz, N., Olivier, B., & Steyn, F. (2018). The use of parametric effect sizes in single  
5 study musculoskeletal physiotherapy research: A practical primer. *Phys Ther Sport*,  
6 355 32, 87-97. doi:10.1016/j.ptsp.2018.05.002  
7  
8 356  
9  
10 357 **Perneger, T. V. (1998). What's wrong with Bonferroni adjustments. *BMJ*, 316(7139),**  
11 **1236-1238. doi:10.1136/bmj.316.7139.1236**  
12  
13  
14 359 Piras, I. S., Costa, A., Tirindelli, M. C., Stoccoro, A., Huentelman, M. J., Sacco, R., . . .  
15 360 Lintas, C. (2020). Genetic and epigenetic MTHFR gene variants in the mothers of  
16 attention-deficit/hyperactivity disorder affected children as possible risk factors for  
17 361 neurodevelopmental disorders. *Epigenomics*. doi:10.2217/epi-2019-0356  
18  
19 362  
20  
21  
22 363 Rai, V. (2018). Strong Association of C677T Polymorphism of  
23 364 Methylenetetrahydrofolate Reductase Gene With Nosyndromic Cleft Lip/Palate  
24 (nsCL/P). *Indian J Clin Biochem*, 33(1), 5-15. doi:10.1007/s12291-017-0673-2  
25 365  
26  
27  
28 366 Raut, J. R., Simeone, R. M., Tinker, S. C., Canfield, M. A., Day, R. S., & Agopian, A.  
29 367 J. (2019). Proportion of Orofacial Clefts Attributable to Recognized Risk Factors.  
30 368 *Cleft Palate Craniofac J*, 56(2), 151-158. doi:10.1177/1055665618774019  
31  
32  
33  
34 369 Reutter, H., Birnbaum, S., Lacava, A. D., Mende, M., Henschke, H., Berge, S., . . .  
35 370 Mangold, E. (2008). Family-based association study of the MTHFR polymorphism  
36 C677T in patients with nonsyndromic cleft lip and palate from central Europe. *Cleft*  
37 371 *Palate Craniofac J*, 45(3), 267-271. doi:10.1597/06-174  
38 372  
39  
40  
41 373 Richmond, R. C., & Joubert, B. R. (2017). Contrasting the effects of intra-uterine  
42 374 smoking and one-carbon micronutrient exposures on offspring DNA methylation.  
43 375 *Epigenomics*, 9(3), 351-367. doi:10.2217/epi-2016-0135  
44  
45  
46  
47 376 Shaw, G. M., Todoroff, K., Finnell, R. H., Rozen, R., & Lammer, E. J. (1999). Maternal  
48 377 vitamin use, infant C677T mutation in MTHFR, and isolated cleft palate risk. *Am J*  
49 378 *Med Genet*, 85(1), 84-85.  
50  
51  
52  
53 379 Sinclair, K. D., Allegrucci, C., Singh, R., Gardner, D. S., Sebastian, S., Bispham, J., . . .  
54 380 . Young, L. E. (2007). DNA methylation, insulin resistance, and blood pressure in  
55 offspring determined by maternal periconceptional B vitamin and methionine status.  
56 381 *Proc Natl Acad Sci U S A*, 104(49), 19351-19356. doi:10.1073/pnas.0707258104  
57  
58 382  
59  
60

- 1  
2  
3  
4 383 Stock, N. M., Humphries, K., Pourcain, B. S., Bailey, M., Persson, M., Ho, K. M., . . .  
5  
6 384 Sandy, J. (2016). Opportunities and Challenges in Establishing a Cohort Study: An  
7  
8 385 Example From Cleft Lip/Palate Research in the United Kingdom. *Cleft Palate*  
9  
10 386 *Craniofac J*, 53(3), 317-325. doi:10.1597/14-306
- 11  
12 387 Stover, P. J. (2009). One-carbon metabolism-genome interactions in folate-associated  
13  
14 388 pathologies. *J Nutr*, 139(12), 2402-2405. doi:10.3945/jn.109.113670
- 15  
16 389 van der Put, N. M., Gabreels, F., Stevens, E. M., Smeitink, J. A., Trijbels, F. J., Eskes,  
17  
18 390 T. K., . . . Blom, H. J. (1998). A second common mutation in the  
19  
20 391 methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube  
21  
22 392 defects? *Am J Hum Genet*, 62(5), 1044-1051. doi:10.1086/301825
- 23  
24 393 Xuan, Z., Zhongpeng, Y., Yanjun, G., Jiaqi, D., Yuchi, Z., Bing, S., & Chenghao, L.  
25  
26 394 (2016). Maternal active smoking and risk of oral clefts: a meta-analysis. *Oral Surg*  
27  
28 395 *Oral Med Oral Pathol Oral Radiol*, 122(6), 680-690.  
29  
30 396 doi:10.1016/j.oooo.2016.08.007
- 31  
32 397 Zhou, Y., Sinnathamby, V., Yu, Y., Sikora, L., Johnson, C. Y., Mossey, P., & Little, J.  
33  
34 398 (2020). Folate intake, markers of folate status and oral clefts: An updated set of  
35  
36 399 systematic reviews and meta-analyses. *Birth Defects Res*, 112(19), 1699-1719.  
37  
38 400 doi:10.1002/bdr2.1827
- 39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60